CHARGE DENSITY ON THE INNER SURFACE OF PEA THYLAKOID MEMBRANES

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1. Introduction

The existence of electrical charges on the surface of chloroplast thylakoid membranes has important implications from a number of points of view (review [1]). To date, however, most of our knowledge of the extent and origin of these charges is restricted to the outer thylakoid surface. This is unsatisfactory since surface electrical effects include transmembrane phenomena which require information about charge densities on both the outer and inner surfaces [1,2]. Some attempts, employing indirect methods, have been used to estimate the charge density on the inner thylakoid surface in the vicinity of the photosystem two [3] and photosystem one [4] electron donation sites. Here, we extend these studies using inside-out thylakoid vesicles isolated as in [5].

2. Materials and methods

'Inside-out' and 'Right-side-out' thylakoid vesicles were prepared from chloroplasts isolated from pea (Feltham first) as in [5]. The chloroplasts were isolated as in [6] and were incubated, on ice, for 30 min in 'high salt phosphate' buffer (250 mM Na^+) then subjected to Yeda press treatment. The granal pellet obtained after centrifugation (35 000 × *g*) of the fragmented membranes was resuspended in low salt phosphate buffer (25 mM Na^+) before further Yeda press treatment. The resulting mixed vesicle population was separated by phase partition using a 50 g phase system consisting of 6.0% dextran (w/w), 6.0% polyethylene glycol (w/w), 5 mM NaCl, 10 mM Na_2HPO_4 (pH 7.4), 20 mM sucrose and fragmented chloroplasts at 0.4 mg chl/g. These constituents were well mixed at 2°C then subjected to centrifugation for 3 min at 1000 × *g*; the top and bottom phases were then repartitioned twice more and the resulting membrane vesicles collected by diluting the final phases with 5 × 10 mM NaCl and subjecting them to centrifugation (30 min at 35 000 × *g*).

For 9-amino acridine fluorescence measurements, samples were resuspended and assayed in 0.1 M sorbitol, 1 mM Hepes (pH 7.5 with 0.43 mM KOH). The method used for measuring the fluorescence and the principles and assumptions underlying the calculations have been detailed in [7,8]. Vesicles equivalent to 20 μg chl/ml were used and 9-amino acridine was 10 μM.

Electrophoretic mobility studies were accomplished using a Zeiss cytopherometer as in [9]. For measurement, the membrane fragments equivalent to 50 μg chl/ml were suspended in either 6.0% sorbitol, 20 mM KCl, 10 mM Tris–HCl at pH 8.0 or in 6.0% sorbitol, 20 mM KCl, 10 mM acetate–Tris at pH 4.0. The pH of the acetate buffer was adjusted with Tris or HCl to determine the isoelectric points of the vesicles, with no apparent effect on the conductance of the solution.

3. Results

Fig.1 shows light-induced pH changes measured with suspensions of inside-out (B3) and right-way-out (T3) membranes. Unlike the T3 vesicles, the B3 membranes pumped H^+ out. Both types of pH changes were inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The membranes were subjected to particle electrophoresis and recorded mobilities are given in table 1 where they are compared with that

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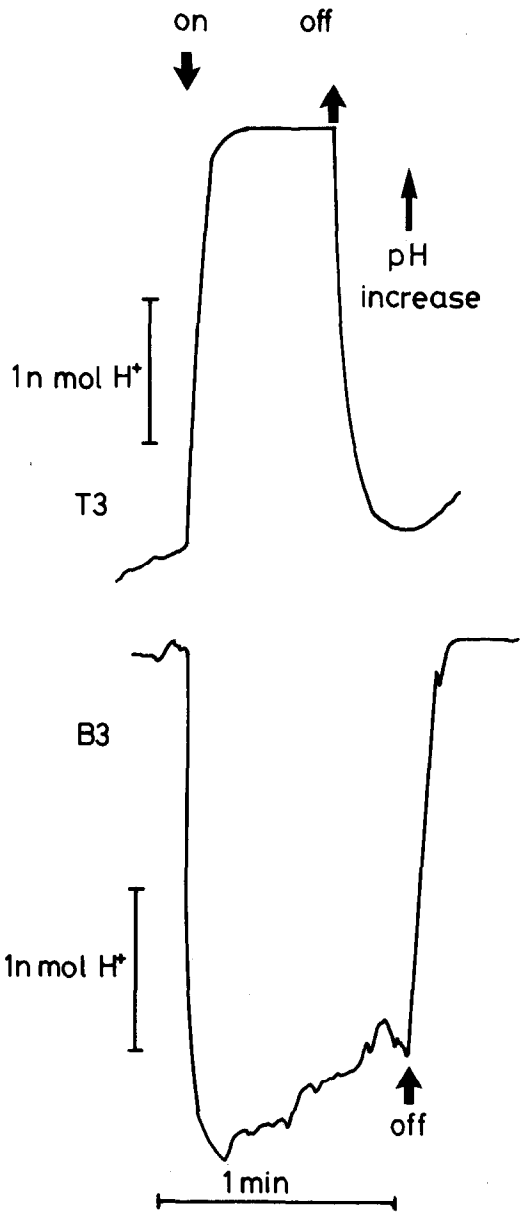

Fig.1. Light-induced reversible proton fluxes of inside-out (B3) and right-way-out (T3) vesicles. The assay medium consisted of 0.5 mM *p*-benzoquinone as electron acceptor, 10 mM NaCl and vesicles corresponding to 100 μg chl. (1 M, total vol. 2 ml). The medium was adjusted to pH 6.5 with NaOH before use. The extent of proteon flux was determined by adding known quantities of NaOH and of HCl.

of intact isolated thylakoids. At pH 8.0 the B3 vesicles diffused more rapidly in the electric field (towards the anode) than did T3 vesicles or intact thylakoid membranes. Also the 3 different membrane systems had different isoelectric points as judged from the value of the external pH which gave rise to zero electrophoretic mobility. These values are in line with the measurements in [9,10].

When B3 or T3 vesicles were suspended in a low salt containing medium in the presence of 9-amino acridine, the fluorescence yield of the dye was low. Addition of either mono- or divalent cations (K^+ and Mg^{2+}, respectively) gave rise to a significant increase in the 9-amino acridine fluorescence yield. The increase depended on the concentration of inorganic cation added as shown in fig.2. In agreement with similar studies using intact isolated thylakoids [7,8] the increase in 9-amino acridine fluorescence was also highly dependent on the valency of the cation added (see fig.2 and table 1). These findings have been explained in terms of electrostatic theory and can be exploited as a means of estimating surface charge densities [7,8]. It has been argued that the 9-amino acridine level is a reflection of the surface potential (ψ_o). Thus, when the fluorescence quenching is at the same level in the presence of either mono- or di-valent cations, then the surface potential values are identical and the surface charge density can be calculated in the manner shown below.

Table 1
Electrophoretic mobility measurements

	Electrophoretic mobility ($cm^2 . V^{-1} . s^{-1}$)	σ ($\mu C/cm^2$)	Isoelectric point
Intact thylakoids	$-1.52 \pm 0.08 \times 10^{-4}$ (3)	-1.09 ± 0.04 (3)	4.3
T3	$-1.87 \pm 0.28 \times 10^{-4}$ (3)	-1.30 ± 0.17 (3)	4.5
B3	$-2.19 \pm 0.30 \times 10^{-4}$ (3)	-1.50 ± 0.19 (3)	4.0

Results ± standard deviation (number of samples)

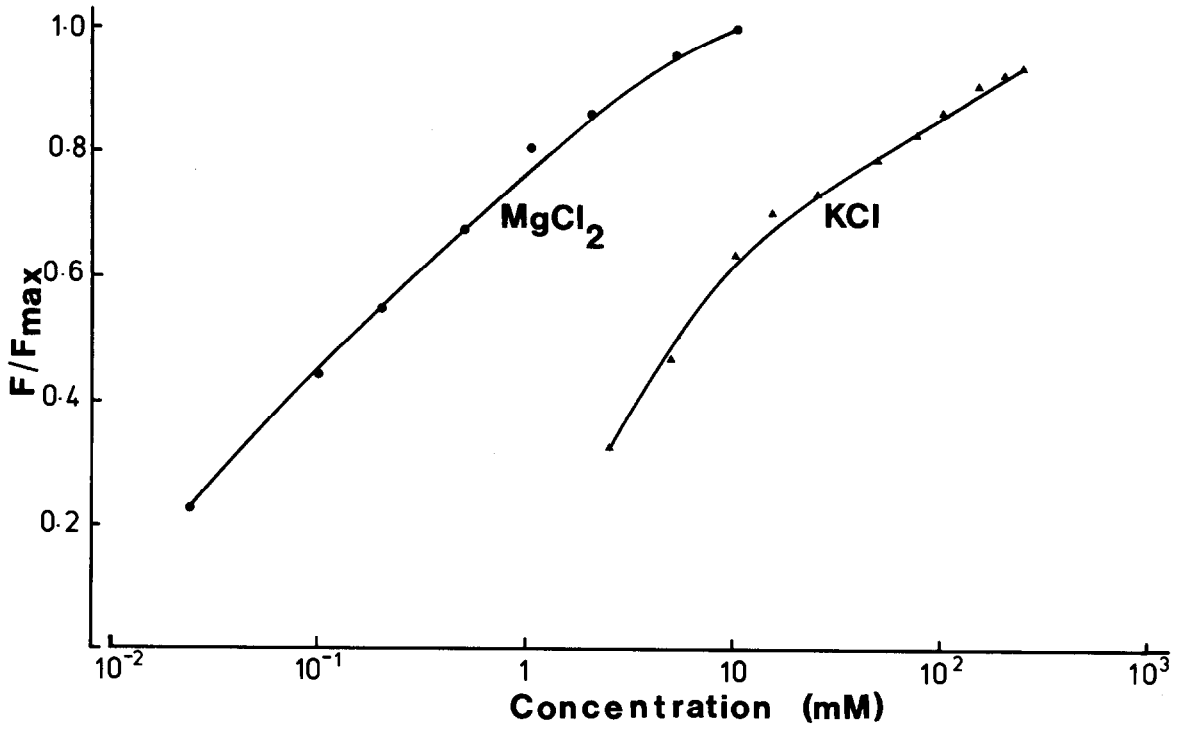

Fig.2. The effect of mono- and divalent cations on 9-amino acridine fluorescence in the presence of inside-out thylakoid vesicles. Assay conditions are described in the text. F_{max} is the maximum fluorescence intensity observed on adding 20 mM $MgCl_2$.

4. Calculations and discussion

The electrophoresis studies clearly indicate that B3 vesicles are more negatively charged than T3 vesicles. However, both types of membranes carry a higher surface charge density than intact thylakoids. The mobility value obtained for the thylakoids is similar to that reported previously and has been used to calculate surface charge densities by applying the Smoluchowski equation [9]:

$$u = \frac{\epsilon_r \epsilon_o}{\eta} \zeta \qquad (1)$$

where u is the electrophoretic mobility, ϵ_r is the relative permittivity of the solution, ϵ_o is the permittivity of a vacuum, η is the dynamic viscosity of the medium and ζ is the zeta potential. The ζ potential is the electrical potential at the hydrodynamic plane of shear. Limitations and assumptions which underlie the application of this equation have been discussed in [1,9]. Bearing in mind the short-comings of the approach, the ζ potential can be obtained from the experimental data given in table 1. Assuming that the ζ potential is equal to the surface potential (ψ_o) it is possible to calculate the surface charge density (σ) by using the Poisson–Boltzmann relation:

$$\sigma = \mp [2\, \epsilon_r \epsilon_o\, RT \sum_i C_{ib} (\exp\{-Z_i F\psi_o/RT\} - 1)]^{1/2} \qquad (2)$$

where C_{ib} is the concentration of the i-th ion in the bulk of the suspension medium, F is the Faraday and R is the gas constant.

Using eq. (1) and (2) gives the surface charge density values shown in table 1. These values are almost certainly underestimated because of the assumption that $\zeta = \psi_o$ [1,11]. Also the technique tends to give an 'average potential' and does not give information about localized charge densities resulting from the heterogeneity of the surface charge distribution. For this reason, the technique of using 9-amino acridine may have some advantages [8]. The data given in fig.2 and table 2 can be used to calculate σ values for B3 and T3 vesicles by solving eq. (2) simultaneously for conditions when a divalent salt (C'')

Table 2
9-amino acridine fluorescence measurements

	$MgCl_2$ (mM)	KCl (mM)	σ ($\mu C/cm^2$)
T3	0.120 ± 0.03 (3)	4.20 ± 0.80 (3)	−2.06 ± 0.17 (3)
B3	0.113 ± 0.002 (4)	6.93 ± 1.80 (4)	−3.67 ± 0.50 (4)

Results ± standard deviation (number of samples). Concentration of salts given for 50% release of fluorescence quenching (F/F_{max} = 50%)

gives the same relief of 9-amino acridine fluorescence quenching as a monovalent salt (C'). If it is assumed that under these conditions the surface potentials are identical, and ignoring background ionic levels (which were very low), then the following equation is derived from eq. (2) for symmetrical electrolytes:

$$\sigma = -[2\,\epsilon_r\epsilon_o\,RT\,(C'^2 - 4C'C''/C'')]^{1/2} \quad (3)$$

The values of σ given in table 2 have been calculated using eq. (3) for C' and C'' corresponding to 50% release of 9-amino acridine fluorescence quenching. Although the calculated values of σ are greater than those obtained from the electrophoretic measurements, they confirm that the surface of the inside-out B3 membranes carry more net negative charge than that of the T3 membranes. The higher values of σ obtained by the 9-amino acridine technique compared with those calculated from the electrophoresis data is consistent with the idea that 9-amino acridine is a more intimate probe of the surface electrical charge, a conclusion which was also drawn from earlier measurements with intact thylakoid membranes [7,8].

It has been argued that the B3 vesicles are derived from the partition region of the grana and contain mainly photosystem two (PS2) components [12,13]. The σ value of $-3.67\ \mu C/cm^2$ calculated for the inner thylakoid surface in the vicinity of PS2 is thus in agreement with the value estimated using electron paramagnetic resonance spectroscopy [3]. For the T3 vesicles, the value of $-2.06\ \mu C/cm^2$ compares well with that used for the average charge density of the outer surface of intact thylakoid membranes [1,14] and in agreement with a value recently calculated from electron transport studies [3]. However, it has been argued that the net electrical charge density on the outer surface of the appressed membranes of the grana is low so as to allow the two adjacent membrane surfaces to approach each other closely [1,15]. If this argument is correct, and if the appressed membranes contain mainly PS2, then it must be concluded that there is a significant asymmetry of surface charge near to the PS2 reaction centre greater than that previously thought [3]. Such an asymmetry could play an important role in the stabilization of charge separation across the membrane [16].

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References

[1] Barber, J. (1980) Biochim. Biophys. Acta 594, 253–308.
[2] Barber, J. (1982) Annu. Rev. Plant Physiol. in press.
[3] Yerkes, C. and Babcock, G. T. (1981) Biochim. Biophys. Acta 634, 19–29.
[4] Itoh, S. (1979) Biochim. Biophys. Acta 548, 579–595.
[5] Andersson, B. and Åkerlund, H. E. (1978) Biochim. Biophys. Acta 503, 462–472.
[6] Nakatani, H. Y. and Barber, J. (1977) Biochim. Biophys. Acta 461, 510–512.
[7] Chow, W. S. and Barber, J. (1980) Biochim. Biophys. Acta 591, 82–91.
[8] Chow, W. S. and Barber, J. (1980) J. Biochem. Biophys. Methods 3, 173–185.
[9] Nakatani, H. Y., Barber, J. and Forrester, J. A. (1978) Biochim. Biophys. Acta 504, 215–225.
[10] Åkerlund, H. E., Andersson, B., Persson, A. and Albertsson, P. A. (1979) Biochim. Biophys. Acta 552, 238–246.
[11] Haydon, D. A. (1961) Biochim. Biophys. Acta 50, 450–457.
[12] Andersson, B. and Anderson, J. M. (1980) Biochim. Biophys. Acta 593, 427–460.
[13] Anderson, J. M. (1981) FEBS Lett. 124, 1–10.
[14] Barber, J., Mills, J. and Love, A. (1977) FEBS Lett. 74, 174–181.
[15] Barber, J. (1980) FEBS Lett. 118, 1–10.
[16] Duniec, J. T. and Thorne, S. W. (1981) FEBS Lett. 126, 1–4.